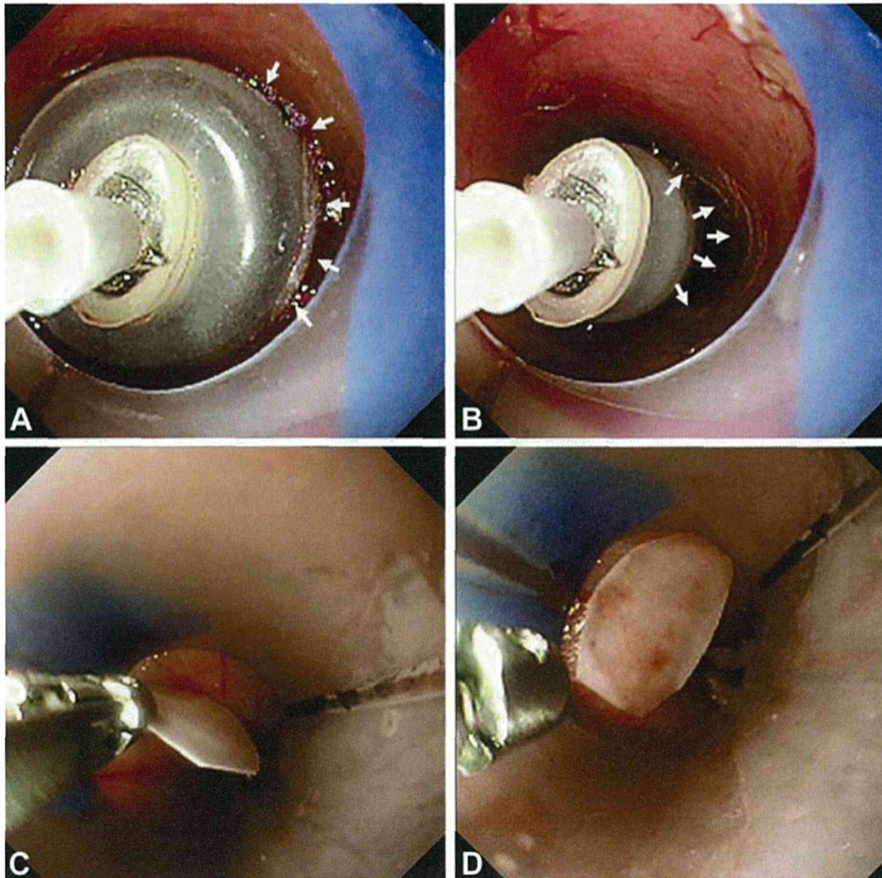

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Figure 2. Endoscopic views of a cell sheet transplantation. **A, B,** The cell sheet transplantation device method. After delivery of the cell sheet through an overtube to the ulcer site, the positions of the cell sheet and ulcer site were aligned by insertion/extraction and rotation of the device. The balloon was then inflated and attached the cell sheet to the ulcer site for approximately 30 seconds. The balloon was then deflated and the cell sheet was successfully transplanted. **C, D,** The conventional support membrane method. The support membrane with a cell sheet was carefully attached to the ulcer site by using conventional endoscopic forceps. The transplantation procedure was completed after pushing several points of the back side of the support membrane and waiting for more than 5 minutes to confirm that the support membrane did not become detached.

deflation, the cell sheet was confirmed to be transplanted. Five other cell sheets were also successfully transplanted in a similar manner with no adverse events.

The conventional support membrane method is shown in Figures 2C and 2D. A round support membrane with a cell sheet was grasped and delivered to the ESD site through an overtube; it was then attached and the membrane was immediately pushed several times. After confirming no detachment for more than 5 minutes, the endoscope was withdrawn.

Macroscopic analysis: transplantation success rate and area

~~Macroscopic views of the ESD sites are shown in~~ Q8 ~~Figure 6.~~ The statistical analysis of the transplantation success rate and area is summarized in Table 2.

Histological analysis

The histology of the cell sheets transplanted by the device Q9 is shown in Figure ~~7~~ 3. The basal layers of the cell sheets, which expressed integrin β4, p63, and CK14, were adhered to the host tissue.

DISCUSSION

The cell sheet transplantation device provides stable and easy transfer of therapeutic materials by storing them inside by balloon deflation, and is assumed to deploy conventional polyglycolic acid sheet[16,17] or gel[18,19] for preventing perforation or bleeding after ESD.

In this first feasibility study, the device enabled successful transplantation of almost all cell sheets onto a larger area in a shorter time with no adverse events than with the conventional method. Adhesion of the basal layers of transplanted cell sheets onto the ulcer site was histologically confirmed. These results support the feasibility of the device. Furthermore, the device might improve the effectiveness of cell sheet therapy because transplantations in larger areas would be more efficient for preventing stenosis in our experience (Kanai et al, manuscript in preparation).

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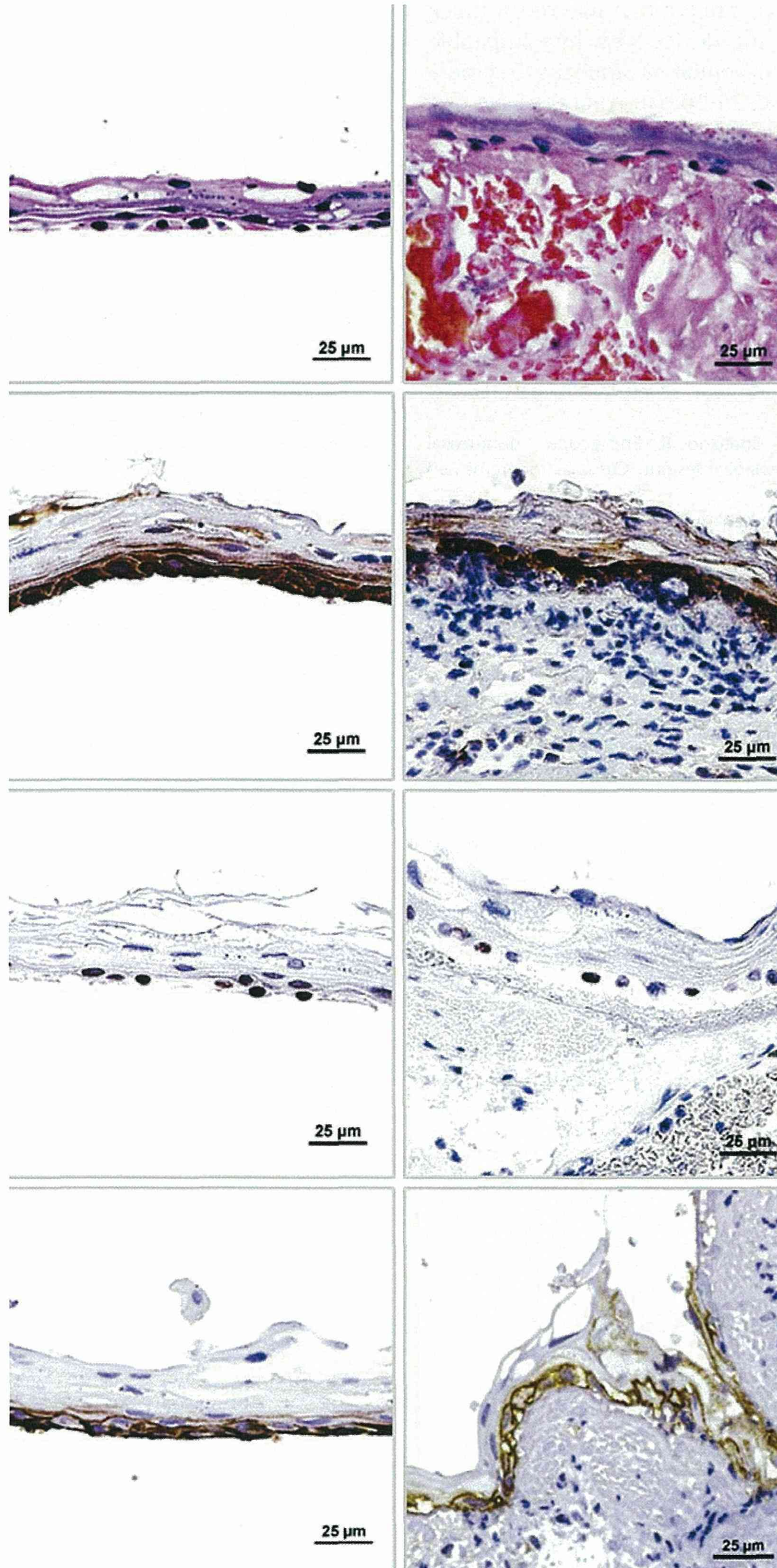

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Figure 3. Histology of fabricated cell sheets and transplanted cell sheets by using the transplantation device 6 hours after transplantation (orig. Q14 mag. ×40). The basal cell epithelium of a transplanted cell sheet using the device was confirmed to adhere to the host tissue while maintaining the basic components of cell sheets, such as cell–cell junctions and integrin expression.

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We used a 3-dimensional printer that allowed a quick turnaround for improving the device with biocompatible material. ~~We believe the potential of applying 3-dimensional printed plastic devices in human clinical trials~~.

In conclusion, a novel endoscopic device for cell sheet transplantation was designed. Improvements are now in progress for a human clinical study.

REFERENCES

1. Repici A, Hassan C, Carlino A, et al. Endoscopic submucosal dissection in patients with early esophageal squamous cell carcinoma: result from a prospective western series. Gastrointest Endosc 2010;71:715-21.
2. Hammad H, Kaltenbach T, Soetikno R. Endoscopic submucosal dissection for malignant esophageal lesions. Curr Gastroenterol Rep 2014;16:386.
3. Wen J, Yang Y, Liu Q, et al. Preventing stricture formation by covered esophageal stent placement after endoscopic submucosal dissection for early esophageal cancer. Dig Dis Sci 2014;59:658-63.
4. Ono S, Fujishiro M, Niimi K, et al. Long-term outcomes of endoscopic submucosal dissection for superficial esophageal squamous cell neoplasms. Gastrointest Endosc 2009;70:860-6.
5. Lee WJ, Jung HY, Kim do H, et al. Intralesional steroid injection to prevent stricture after near-circumferential endoscopic submucosal dissection for superficial esophageal cancer. Clin Endosc 2013;46:643-6.
6. Yamaguchi N, Isomoto H, Nakayama T, et al. Usefulness of oral prednisolone in the treatment of esophageal stricture after endoscopic submucosal dissection for superficial esophageal squamous cell carcinoma. Gastrointest Endosc 2011;73:1115-21.
7. Lee M, Kubik CM, Polhamus CD, et al. Preliminary experience with endoscopic intralesional steroid injection therapy for refractory upper gastrointestinal strictures. Gastrointest Endosc 1995;41:598-601.
8. Tietjen T, Pasricha P, Kalloo A. Management of malignant esophageal stricture with esophageal dilation and esophageal stents. Gastrointest Endosc Clin N Am 1994;4:851-62.
9. Verschuur EM, Homs MY, Steyerberg EW, et al. A new esophageal stent design (Niti-S stent) for the prevention of migration: a prospective study in 42 patients. Gastrointest Endosc 2006;63:134-40.
10. Ohki T, Yamato M, Murakami D, et al. Treatment of oesophageal ulcerations using endoscopic transplantation of tissue-engineered autologous oral mucosal epithelial cell sheets in a canine model. Gut 2006;55:1704-10.
11. Kanai N, Yamato M, Ohki T, et al. Fabricated autologous epidermal cell sheets for the prevention of esophageal stricture after circumferential ESD in a porcine model. Gastrointest Endosc 2012;76:873-81.
12. Yamada N, Okano T, Sakai H, et al. Thermo-responsive polymeric surfaces; control of attachment and detachment of cultured cells. Makromol Chem Rapid Commun 1990;11:571-6.
13. Yamato M, Konno C, Kushida A, et al. Release of adsorbed fibronectin from temperature-responsive culture surfaces requires cellular activity. Biomaterials 2000;21:981-6.
14. Ohki T, Yamato M, Ota M, et al. Prevention of esophageal stricture after endoscopic submucosal dissection using tissue-engineered cell sheets. Gastroenterology 2012;143 582-588.e1-2.
15. Takagi R, Yamato M, Murakami D, et al. Low calcium culture condition induces mesenchymal cell-like phenotype in normal human epidermal keratinocytes. J Biochem Biophys Res Commun 2011;412:226-31.
16. Ono S, Yosuke T, Mitsuhiro F, et al. An effective technique for deliver y of polyglycolic acid sheet after endoscopic submucosal dissection of the esophagus: the clip and pull method. Endoscopy 2014;46:E44-5.
17. Takimoto K, Toyonaga T, Matsuyama K. Endoscopic tissue shielding to prevent delayed perforation associated with endoscopic submucosal dissection for duodenal neoplasms. Endoscopy 2012;44:E414-5.
18. Mori H, Rafiq K, Kobara H, et al. Steroid permeation into the artificial ulcer by combined steroid gel application and balloon dilatation: prevention of esophageal stricture. J Gastroenterol Hepatol 2013;28: 999-1003.
19. Yang F, Ma D, Cai QC, et al. Esophageal strictures after extensive endoscopic submucosal dissection: steroid gel application, the ideal choice? J Gastroenterol Hepatol 2013;28:1795-7.

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特集4

細胞治療1

自己培養歯根膜細胞シートを用いた歯周組織の再建

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キーワード：細胞シート，自己培養歯根膜細胞シート，歯周組織再生

はじめに

歯周組織再生が困難であることの１つの理由は，歯と歯槽骨をつなぐ付着器官(セメント質-歯根膜-歯槽骨)を丸ごと再生しなくてはならないからである．さらには組織学的解析をしなければ有効性を正確には判定できないことも臨床評価を困難にしている．既存の歯周組織再生療法としては，

①自家・他家・合成物などの骨補填材
②遮断膜
③成長因子などの生物製剤

等がすでに臨床で応用されているが，適応症が限られていたり，予知性が不安定であるなどの問題を包含している．また，これらのマテリアルを用いた再生療法はそもそも直接的ではなく，欠損部に適用したマテリアルが患者自身の細胞に働きかけることで再生を引き起こすという間接的な再生療法である．

「細胞シート」の可能性

そこで，近年では幹細胞生物学と組織工学を背景とした細胞治療の研究が歯周領域においても大学を中心に進められており，われわれは細胞ソースとしては患者の自己歯根膜幹細胞に着目して研究を進めてきた(図１)．また，組織を再構築するためのアプローチとして「細胞シート工学」をコア技術として取り入れている(図２)．「細胞シート」を用いることで，

増殖能の高い歯根膜幹細胞

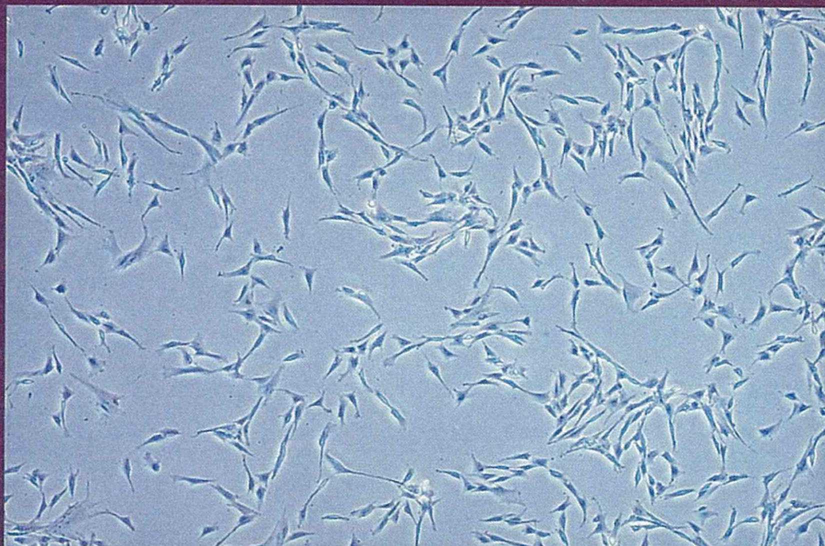

図１　骨髄由来細胞と比べると圧倒的な高い増殖能をもっている．

歯根膜細胞シート

図２　特殊な培養皿(温度応答性培養皿)を用いることで細胞をシート状に回収・移植することができる．

シート状の歯根膜幹細胞を歯周欠損根面に着実に移植することが可能であり，細胞間の結合が保持されているため，移植した細胞が拡散しにくく，着実な細胞移植が可能となる．本テクノロジーは角膜・心筋・食道の再生にすでに使われている．

厚生労働省に提出した書類

図３　厚さ６cm程度の書類を作成し，厚生労働大臣の承認を得たのちに臨床研究を開始することが可能となる．

「自己培養歯根膜細胞シート」の応用

東京女子医科大学では小動物・大動物を用いて「歯根膜細胞シート」の実験室レベルでの安全性・有効性を確認し，「ヒト幹細胞を用いる臨床研究に関する指針」に合致した臨床研究として2011年１月に厚生労働大臣より臨床研究実施の承認を得た(図３)．具体的には患者自身の抜去歯から歯根膜幹細胞を抽出し，「細胞シート工学」を用いてシート状に回収された「自己培養歯根膜細胞シート」を歯周欠損の根面

細胞プロセッシングセンター内の培養風景

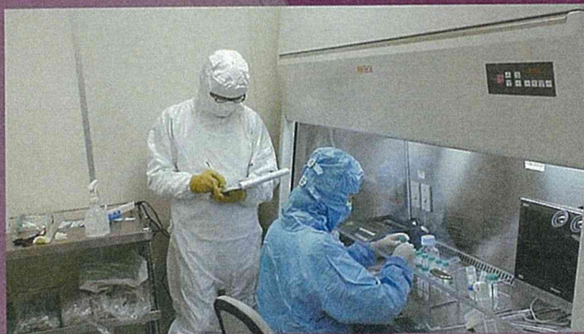

図４　防塵服を二重に着た２人１組で培養を実施する．すべての工程を記録に残す．

細胞プロセッシングセンター内で用いられる紙

図５　防塵加工のされた特殊な紙を滅菌した後に使用する．

臨床例

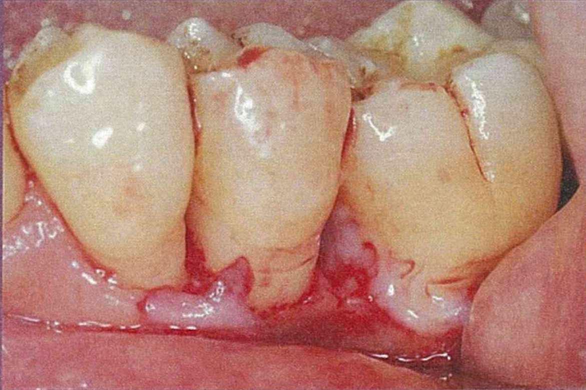

図6　|6 近心に垂直性の骨欠損が観察された．郭清術後にEDTA 処理を行う．

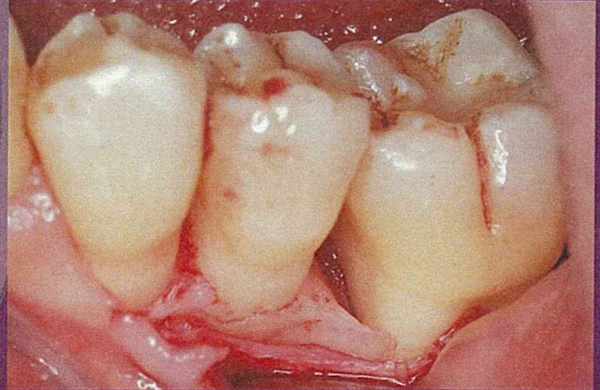

図7　EDTA 処理 2 分後に十分な量の生理食塩水で EDTA を洗い流す．

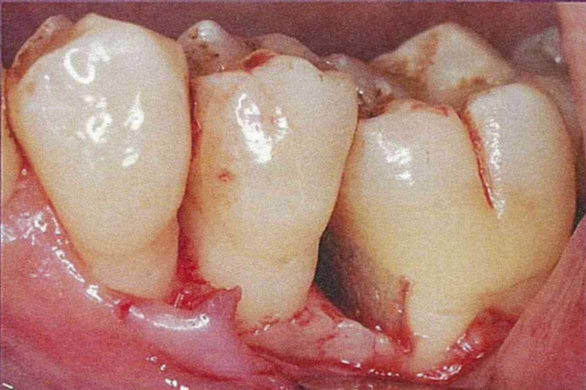

図8　欠損のサイズにトリミングされた歯根膜シートは根面に設置された．

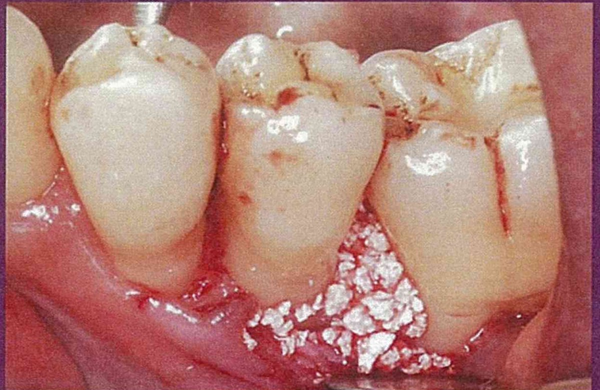

図9　骨欠損には骨補填材を充填する．

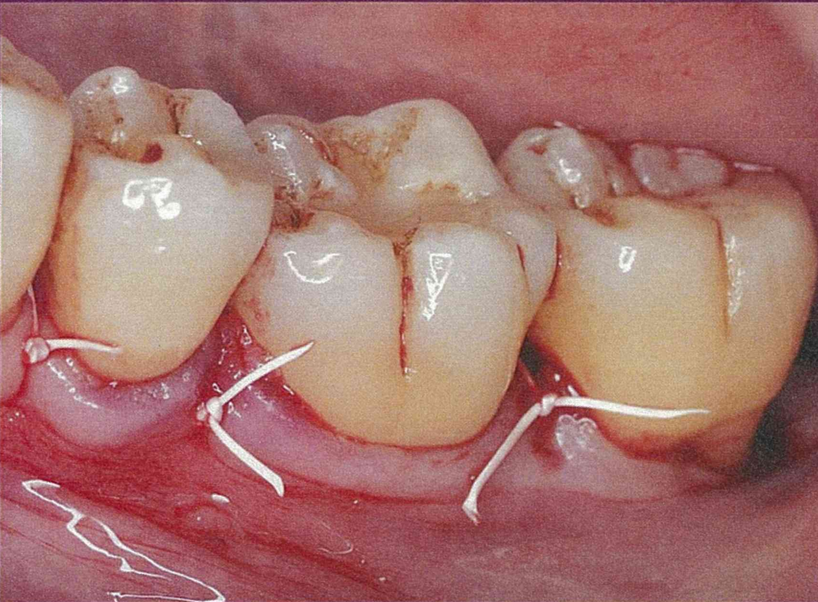

図10　歯肉弁を復位・縫合した状態．

歯周組織再生療法における自己培養歯根膜細胞シートの特徴

使用開始時期	・2008年より研究開発を主に大型動物を用いて実施し，その安全性，有効性が確認された．2011年11月より臨床研究を開始した．
価格	・未定．
特徴	・患者自身の歯根膜に存在する幹細胞を生体外で増幅し，シート状に加工した細胞を歯根面に移植する． ・骨欠損には骨補填材を充填する．
長所	・患者の血清で育てられた患者自身の歯根膜幹細胞を移植しているので安全性が高い． ・欠損サイズにトリミングするだけで，移植術式はきわめて簡便である． ・広汎な欠損への応用が期待されている．
短所	・健全な歯周組織を有する抜去可能な不働歯等がなければ本治療法を享受できない． ・培養期間（約４週間）が必要である． ・コストが高い．
臨床上の注意点	・通法のフラップ手術後に，EDTA による根面処理を実施している．EDTA による細胞死を防ぐために生理食塩水による洗浄を入念に行っている． ・骨補填材は大盛りにすると術後の歯肉弁の治癒が悪化するので，盛りすぎない．
筆者からのアピールポイント	・本治療法は研究開発段階ではあるが，今までのところ安全性と優れた有効性が確認されている．新しい治療オプションの１つとなるべく研究を続けており，一般普及をめざしたい．

に移植する臨床研究を進めている．無菌的に細胞を培養できる「細胞プロセッシングセンター」と呼ばれる特別な施設（図４，５）で作製された細胞シートは３層に重ね合わされ，郭清術の行われた歯周欠損の歯根面に設置され，骨欠損にはβ-リン酸三カルシウム（オスフェリオン：オリンパス）を充填することで付着器官の再生を促す．現在までに８例の移植が終了し，順調な経過を示しており，今後２年間で全10症例の臨床試験を完了する予定である（図６～10）．

謝辞

本研究を遂行するにあたり東京女子医科大学・先端生命医科学研究所の葭田敏之研究員，辰巳公平助教，黒田ほずえ技師，高木亮博士研究員，加藤ゆか先生，森南奈先生（福岡歯科大学・歯周病より国内留学），また東京女子医科大学・歯科口腔外科の貝渕信之先生，岡本俊宏准教授，扇内秀樹名誉教授，医局員の皆様，さらに東京医科歯科大学の和泉雄一教授，木下淳博教授，妻沼有香先生，山田梓先生，Supreda Suphanantachat 先生，鬼塚理先生，矢野孝星先生からは多大なご支援・ご協力を賜った．またセルシード社・オリンパス社にもご協力していただいた．本研究は先端融合領域イノベーション創出拠点形成プログラム「再生医療本格化のための最先端技術融合拠点」ならびに G-COE「再生医療本格化のための集学的教育研究拠点」によって行われた．

Chapter 1

Cell Sheet Engineering for Periodontal Regeneration

Takanori Iwata, Kaoru Washio, Toshiyuki Yoshida,
Isao Ishikawa, Tomohiro Ando,
Masayuki Yamato and Teruo Okano

Additional information is available at the end of the chapter

1. Introduction

Periodontitis is a world-wide infectious disease that destroys the tooth-supporting attachment apparatus, which consists of alveolar bone, cementum, and periodontal ligament. Recent studies have reported numerous associations between periodontitis and systemic diseases, such as cardiovascular disease (de Oliveira et al., 2010) and diabetes mellitus (Lalla and Papapanou, 2011), as well as a higher risk of preterm low birth-weight babies (Offenbacher et al., 1996). Furthermore, researches have recently shown that Bisphosphonate-Related Osteonecrosis of the Jaws (BRONJ) is also associated with severe periodontitis (Vescovi et al., 2011). Therefore, periodontal treatment may not only contribute to oral hygiene but also improvement of systemic conditions (Seymour et al., 2007). Conventional treatments, such as scaling, root-planing, and surgical cleaning, have been performed to remove the bacteria and contaminated tissue. However, these procedures frequently result in the formation of a weak attachment, a condition termed "long junctional epithelium (LJE)" (Caton et al., 1980), wherein the patients tend to present with a recurrence of disease without maintenance therapies (Axelsson and Lindhe, 1981). To overcome this problem, various regenerative therapies, such as guided tissue regeneration (GTR) and enamel matrix derivative, have been introduced in clinical practice. The use of cell-occlusive membranes for GTR is regarded as the first generation of periodontal regeneration, whereas the development and use of growth factors and endogenous regenerative technology for periodontal regeneration is regarded as the second generation of periodontal regeneration (Ishikawa et al., 2009). However, the outcomes of these studies were limited and associated with poor clinical predictability (Esposito et al., 2009). Therefore, stem cell-based approaches for periodontal regeneration have been studied and translated into clinical settings as the third

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generation. In this chapter, we would like to describe the principles of "Cell Sheet Engineering" and its application of clinical settings, featuring our recent translational research for periodontal regeneration.

2. "Cell Sheet Engineering (CSE)"

The cell delivery for periodontal regeneration is usually performed with the combination use of cells and scaffolds, although the location and the differentiation of transplanted is difficult to control. In contrast to approaches that utilize scaffolds, we have developed an alternative technology for cell transplantation using temperature responsive culture dishes, which we call "Cell Sheet Engineering".

2.1. Intelligent surface of *N*-isopropylacrylamide (PIPAAm) and fabrication of cell sheets

Poly(*N*-isopropylacrylamide) (PIPAAm) is a temperature responsive polymer that has been widely utilized for novel biomedical applications. We have developed a PIPAAm-grafted surface as a smart biointerface wherein cell attachment/detachment can be easily controlled by simply changing the temperature (Okano et al., 1995; Yamada et al., 1990). This surface is slightly hydrophobic under cell culture conditions of 37 °C, but readily becomes hydrated and hydrophilic below its lower critical solution temperature (LCST) of 32 °C. Cells can adhere, spread, and proliferate similarly to that on ungrafted tissue culture grade polystyrene surfaces at 37 °C (Figure 1A), and cells detach from the surface by reducing temperature below LCST, making it possible to harvest the cells from the culture surfaces without the use of proteolytic enzymes (Figure 1B).

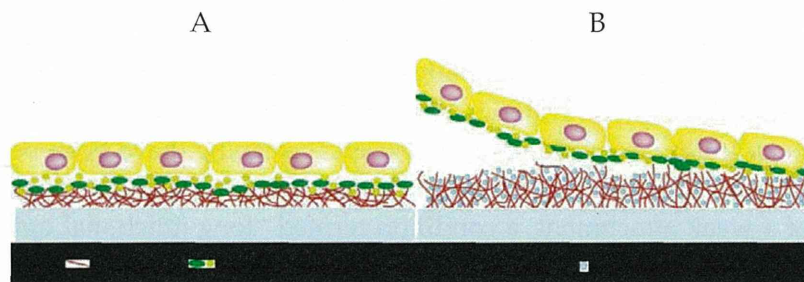

Figure 1. The principle of "Cell Sheet Engineering".

A: Cells can attach and proliferate on grafted surface of the temperature responsive polymer (poly (N-isopropylacrylamide: PIPAAm) at 37 °C, wherein PIPAAm is extensively dehydrated and compact. B: At temperatures below 32 °C, cells with extracellular matrix proteins spontaneously detach from the temperature responsive culture dishes, wherein PIPAAm is fully hydrated with an extended-chain conformation. A simple temperature change can control cell attachment/detachment without any damages. Modified and reprint from Iwata et al., 2013.

The application of this technology has enabled the retrieval of confluently cultured cells, such as keratinocytes (Yamato et al., 2001), corneal epithelial cell sheets (Nishida et al., 2004a), and oral mucosal epithelial cells (Ohki et al., 2006) in the form of a "cell sheet". The epithelial cell sheets are multi-layered and preserve the integrity of proteins such as E-cadherin and laminin 5 that are typically destroyed in the process of enzymatic treatments (Yamato et al., 2001). In addition, recent studies revealed that epithelial cell sheets can be fabricated using temperature responsive culture inserts without feeder layers (Murakami et al., 2006a; b), thereby eliminating exclude xenogeneic factors for animal-free cell transplantation (Takagi et al., 2011).

To fabricate thick tissues, cell sheets can be stacked in layers because they can connect to one another very quickly. A study demonstrated that bilayer cardiomyocyte sheets were completely coupled 46 ± 3 min (mean ± SEM) after the initial layering (Haraguchi et al., 2006), suggesting that multi-layered cell sheets can communicate and become synchronized as functional tissues. Based on this study, multi-layered transplantation was performed (Shimizu et al., 2006b). When more than three cardiomyocyte sheets were layered and transplanted into the subcutaneous space in rats, the appearance of fibrosis and disordered vasculature indicated the presence of fibrotic areas within the transplanted laminar structures. Although the rapid establishment of microvascular networks occurred within the engineered tissues, this formation of new vessels did not rescue the tissues when the thickness was above 80 μm. Using a multiple-step transplantation protocol at 1 or 2 day intervals resulted in rapid neovascularization of the engineered myocardial tissues with a thickness of more than 1 mm (Shimizu et al., 2006b), and these results led us to fabricate prevascularized cell sheets (Sekine et al., 2011). Recent studies demonstrate that the combination of different types of cells, for example an endothelial cell sheet sandwiched with other types of cell sheets, can lead to pre-vascularization *in vitro*, which may allow the graft to survive and function (Haraguchi et al., 2012; Pirraco et al., 2011). Furthermore, the three-dimensional manipulation of fibroblast cell sheets and micro-patterned endothelial cells with a gelatin-coated stacking manipulator produced microvascular-like networks within a 5-day *in vitro* culture (Tsuda et al., 2007). Non-patterned endothelial cell sheets and other types of cell sheets with a fibrin gel manipulator can also produce pre-vascular networks both *in vitro* (Asakawa et al., 2010) and *in vivo* (Sasagawa et al., 2009).

2.2. Cell sheet transplantation in animal models

From the beginning of the 21st century, various types of cells have been extracted, cultured in temperature responsive dishes, and fabricated as cell sheets. Transplantation has been performed, and the efficacy of these cell sheets was evaluated in most of the studies.

2.2.1. Corneal regeneration

Limbal stem-cell deficiency by ocular trauma or diseases causes corneal opacification and loss of vision. To recruit limbal stem cells, a novel cell-sheet manipulation technology that takes advantage of temperature responsive culture surfaces was developed (Nishida et al., 2004a). The results reveal that multi-layered corneal epithelial cell sheets were successfully

fabricated and that their characteristics were similar to those of native tissues. Transplantation of these cell sheets induced corneal surface reconstruction in rabbits. For patients who suffer from unilateral limbal stem deficiency, corneal epithelial cell sheets can be cultured from autologous limbal stem cells. When the objective is to repair the bilateral corneal stem cell deficiency, autologous oral mucosal epithelial cells are utilized to create oral mucosal epithelial cell sheets. The cell sheets contain both cell-to-cell junctions and extracellular matrix proteins, and can be transplanted without the use of any carrier substrates or sutures. Therefore, oral mucosal epithelial sheets were examined as an alternative cell source to expand the possibilities of autologous transplantation. Autologous transplantation to rabbit corneal surfaces successfully reconstructed the corneal surface and restored transparency. Four weeks after the transplantation, epithelial stratification was similar to that of normal corneal epithelia, although the keratin expression profile retained characteristics of the oral mucosal epithelium.

2.2.2. Cardiac regeneration

To enhance the function of cardiac tissue, neonatal rat cardiomyocyte sheets were fabricated and examined (Shimizu et al., 2002). When 4 sheets were layered, spontaneous beating of the engineered constructs was observed. When they were transplanted subcutaneously, heart tissue-like structures and neovascularization within the contractile tissues were observed. The long-term survival of pulsatile cardiac grafts was confirmed for more than one year in rats (Shimizu et al., 2006a). Another study was performed to create thick tissue in rats (Shimizu et al., 2006b). However, the thickness limit for the layered cell sheets of subcutaneous tissue was ~80 μm (3 layers). To overcome this limitation, several transplantations of triple-layer grafts were performed, resulting in an approximately 1 mm-thick myocardium with a well-organized microvascular network. Other types of cell sheets were also examined to improve cardiac function. Adipose-derived mesenchymal stem cells in mice (Miyahara et al., 2006) and skeletal myoblasts in dogs, rats, and hamsters (Hata et al., 2006; Hoashi et al., 2009; Kondoh et al., 2006) were transplanted as cell sheets, demonstrating the efficacy of the method for cardiac repair.

2.2.3. Cartilage regeneration

Chondrocyte sheets applicable to cartilage regeneration were prepared using cell sheet manufacturing technique that takes advantage of temperature responsive culture dishes. The layered chondrocyte sheets were able to maintain the phenotype of cartilage and could be attached to sites that exhibited cartilage damage. The cell sheets act as a barrier for preventing the loss of proteoglycan from these sites and for protection against catabolic factors in the joints of rabbits (Kaneshiro et al., 2006).

2.2.4. Esophageal regeneration

With the recent development of endoscopic submucosal dissection (ESD), large esophageal cancers can be removed using a single procedure. However, complications, such as postoperative inflammation and stenosis, frequently occur after an aggressive ESD procedure,

which can considerably affect the quality of life of the patient. Therefore, a novel treatment combining ESD and the endoscopic transplantation of tissue-engineered cell sheets created using autologous oral mucosal epithelial cells, was examined in a canine model (Ohki et al., 2006). The results confirm the efficacy of the novel combination of the endoscopic approach with the potential treatment of esophageal cancers that can effectively enhance wound healing and possibly prevent postoperative esophageal stenosis.

2.2.5. Hepatocyte regeneration

To address the demand for therapeutic benefits for patients suffering from liver disease, the development of new therapeutic applications is crucial. Therefore, hepatic tissue sheets transplanted into the subcutaneous space of mice have been investigated, resulting in the efficient engraftment of the surrounding cells, as well as the formation of a two-dimensional hepatic tissues network, which was stable for more than 200 days (Ohashi et al., 2007). The engineered hepatic cell sheets also showed several characteristics of liver-specific functionality, and the use of bilayered sheets enhanced these characteristics.

2.2.6. Fibroblast sheet transplantation for sealing air leaks

In thoracic surgery, the development of postoperative air leaks is the most common cause of prolonged hospitalization. To seal the lung leakage, use of autologous fibroblast sheets on the defects was demonstrated to be an effective treatment for permanently sealing air leaks in a dynamic fashion in rats (Kanzaki et al., 2007). Using roughly the same procedures, pleural defects were also closed by fibroblast sheets in pigs (Kanzaki et al., 2008).

2.2.7. Mesothelial cells for the prevention of post-operative adhesions

Post-operative adhesions often cause severe complications such as bowel obstruction and abdominopelvic pain. The use of mesothelial cell sheets was investigated to prevent post-operative adhesions in a canine model (Asano et al., 2006). Mesothelial cells were harvested from tunica vaginalis (Asano et al., 2005) and cell sheets were fabricated on a fibrin gel. The results demonstrated that mesothelial cell sheets are effective for preventing post-operative adhesion formation.

2.2.8. Retinal Pigment Epithelial (RPE) cell regeneration

The retinal pigment epithelium (RPE) plays an important role in maintaining the health of the neural retina. RPE cell sheets were fabricated as a monolayer structure with intact cell-to-cell junctions, similar to that of native RPE (Kubota et al., 2006). In the transplantation study, RPE cell sheets attached to the host tissues in the subretinal space were more effective than the use of injected isolated cell suspensions in rabbits (Yaji et al., 2009).

2.2.9. Urothelial regeneration

Augmentation cystoplasty using gastrointestinal flaps may induce severe complications such as lithiasis, urinary tract infection, and electrolyte imbalance. The use of viable, contig-

uous urothelial cell sheets cultured *in vitro* should eliminate these complications. Canine urothelial cell sheets were grown and their structures were shown to be appropriate (Shiroyanagi et al., 2003). Urothelial cell sheets were autografted onto dog demucosalized gastric flaps successfully, with no suturing or fixation, and generated a multi-layered urothelium *in vivo* (Shiroyanagi et al., 2004). The novel intact cell-sheet grafting method rapidly produced native-like epithelium *in vivo*.

2.2.10. Islet regeneration

To establish a novel approach for diabetes mellitus, pancreatic islet cell sheets were fabricated and transplanted in rats (Shimizu et al., 2009). Laminin-5 was coated on temperature responsive dishes to enhance the initial cell attachment, and the presence of specific molecules, such as insulin and glucagon, was also observed in the recipient site.

2.2.11. Thyroid regeneration

For hormonal deficiencies caused by endocrine organ diseases, continuous oral hormone administration is indispensable to supplement the shortage of hormones. To verify the cytotherapeutic approach, cells from rat thyroid were spread on temperature responsive culture dishes, and cell sheets were created (Arauchi et al., 2009). Rats were exposed to total thyroidectomy as hypothyroidism models and received the thyroid cell sheet transplantation 1 week after the total thyroidectomy. The transplantation of the thyroid cell sheets was able to restore the thyroid function 1 week after the cell sheet transplantation and the improvement was observed long after the surgery.

2.3. Cell sheet transplantation in human clinical trials

In Japan, 6 clinical trials using cell sheet engineering technology have been started or have already been completed.

2.3.1. Corneal reconstruction

The first clinical trial of the cell sheet engineering technology involved a corneal reconstruction using autologous mucosal epithelial cells, and the results were published in 2004 (Nishida et al., 2004b). Oral mucosal tissue was harvested from 4 patients with bilateral total corneal stem-cell deficiencies. Subsequently, cells were cultured for two weeks using a mitomycin C-treated 3T3 feeder layer and transplanted directly into the denuded corneal surfaces without sutures. The results demonstrated that complete re-epithelialization of the corneal surfaces occurred, and the vision of all patients was restored. Recently, autologous oral mucosal epithelial cell sheets cultured with UpCell-Insert technology (CellSeed, Tokyo, Japan) without the feeder layer were transplanted into 25 patients for the treatment of corneal limbal epithelial deficiency in France The safety of the products was established during the 360-day follow-up, and the results confirmed its efficacy for reconstructing the ocular surface. (Burillon et al., 2012).

2.3.2. Endoscopic treatment of esophageal ulceration

Using a canine model (Ohki et al., 2006), autologous oral mucosal epithelial cell sheets were fabricated using the UpCell-Insert technology. After performing the esophageal endoscopic submucosal dissection to remove superficial esophageal neoplasms, cell sheets were transplanted, resulting in the complete prevention of stricture formation in patients with partial circumferential resection (Ohki et al., 2009; Ohki et al., 2012).

2.3.3. Improvements in ischemic cardiomyopathy

Autologous myoblast cells from a patient's thigh were fabricated as cell sheets, and these cell sheets were transplanted into end-stage dilated cardiomyopathy patients in need of left ventricular assist systems (Sawa et al., 2012). The myoblastic cell sheets were transplanted into the affected part of the heart in the patients. The first patient was successfully treated and discharged from the hospital without requiring a ventricular assisting device.

2.3.4. Cartilage regeneration

A clinical trial for cartilage regeneration began in 2011 at Tokai University, Japan. In this study, autologous chondrocytes and synoviocytes were co-cultured with the UpCell-Insert technology. After a period of cultivation, co-cultured cell sheets were combined into three layers and transplanted into the cartilage defects of patients.

2.3.5. Nasal mucosa epithelial cell sheet transplantation to the middle ear bone for preventing hearing loss

A clinical trial for preventing hearing loss began in 2014 at The Jikei University, Japan. Autologous nasal mucosa epithelial cell sheets were transplanted to the surface of bone of the middle ear, and inhibit such as the hyperplasy of granulation tissue and bone, and the progression of fibroblast within middle ear cavity, which induce hearing loss after the surgery of otitis media.

3. Periodontal regeneration

Our laboratory started to introduce cell sheet engineering for periodontal regeneration since sometime after 2000. A key event in periodontal regeneration involves the formation of periodontal ligament and cementum complex (MacNeil and Somerman, 1999), which is a thin surface structure that anchors the tooth to the alveolar socket. Several studies have demonstrated that the cell sheet engineering approach can deliver functional cells in the form of a thin layered sheet, wherein the extracellular matrices, cell-cell junctions, and cell-matrix interactions are well-preserved (Kumashiro et al., 2010). Thus, we have attempted to regenerate this periodontal attachment apparatus based on the technology of "cell sheet engineering" (Yang et al., 2007).

3.1. Small animal studies

Human PDL (hPDL) cell sheets were successfully created using temperature responsive dishes, and the characteristics of hPDL cell sheets were investigated (Hasegawa et al., 2005). In this study, explant culture methods were utilized for the primary culture of hPDL cells. The hPDL cell sheets cultured with ascorbic acid were recovered from the culture dishes as a contiguous sheet accompanied by abundant extracellular matrix components, including type I collagen, integrin β1 and fibronectin. Then, hPDL cell sheets were transplanted as cell pellets into a mesial dehiscence model in athymic rats. Four weeks after surgery, newly formed immature fibers with obliquely anchored dentin surfaces were observed in all the experimental sites, whereas no such findings were observed in any control sites (Figure 2).These results suggest that this procedure based upon the principles of cell sheet engineering can be applied to periodontal regeneration.

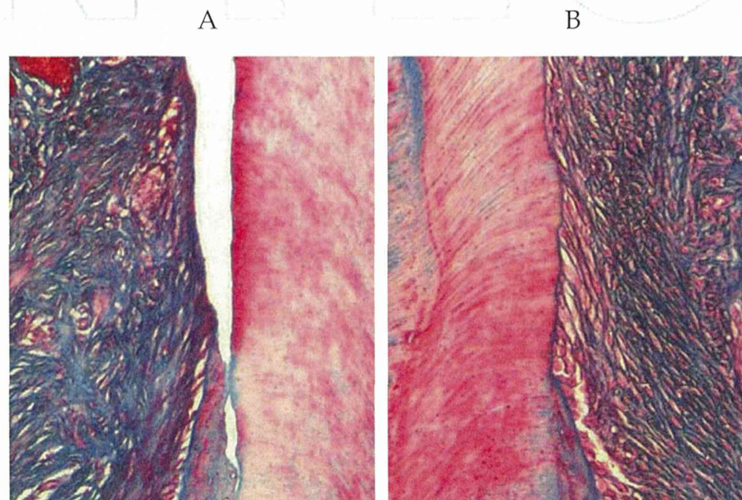

Figure 2. PDL regeneration at 4 weeks postsurgery.

A: Nontransplanted control site. B: hPDL transplanted experimental site. Regeneration of periodontal ligament-like structure was observed only in the experimental site. Azan staining. Modified and reprint from Hasegawa et al., 2005.

Next, the optimal culture condition was examined. Because the osteoinductive medium, which contains 50 μg/ml of ascorbic acid, 10 mM β-glycerophosphate, and 10 nM dexamethasone, enhanced both osteoblastic/cementoblastic and the periodontal differentiation of PDL cells *in vitro,* we compared hPDL cell sheets cultured in the absence and presence of these osteoinductive supplements in a xenogeneic transplantation model (Flores et al., 2008a). Three layered hPDL cell sheets were constructed with fibrin gel and transplanted with a human dentin block into the back of a subcutaneous athymic rat. The constructs were excised for histological investigation 6 weeks after the transplantation. The three-layered hPDL cell sheets-dentin block constructs induced a new cementum-like hard tissue on the surface of the dentin in more than 60% of the samples. Collagen fibers were inserted perpen-

dicularly into the newly formed cementum-like tissue, and this orientation resembled the native Sharpey's fibers. In addition, the regenerative potential of hPDL cell sheets cultured with the osteoinductive medium the was confirmed, when hPDL cell sheets were transplanted onto the root surface of periodontal defects in athymic rat mandibles (Flores et al., 2008b). The results indicate that most of the specimens in the experimental group exhibited a newly-formed cementum and a new attachment of collagen fibers to the cementum layer. No clear cementum layer was observed in the control group (in the absence of osteoinductive supplements). As shown in these experiments, hPDL cells cultured with osteoinductive medium could contribute to the simultaneous regeneration of cementum and PDL.

3.2. Large animal studies

Based on the successful results from small animal studies, we next utilized canine periodontal defect models. Dog PDL (dPDL) cells were extracted using collagenase/dispase digestion. Four individual dPDL cells were successfully isolated and expanded *ex vivo*. Cells were cultured in a standard medium with osteoinductive supplements for 5 days, because longer cultivation induced spontaneous detachment of cell sheets from the UpCell Surfaces. Three-layered dPDL cell sheets were fabiricated with woven polyglycolic acid (PGA) for cell sheet transfer. This PGA product has a number of advantages, including: 1) cell sheets can be easily peeled from temperature responsive dishes, because cell sheets can be attached to the fibers of the woven PGA, 2) the shrinkage of cell sheets can be prevented, 3) easy stacking of multi-layered cell sheets can be achived in a short period of time (see the video attached to the manuscript (Iwata et al., 2009)), 4) easy adjustment of different sizes of cell sheets can be used to cover any defect shape by simply trimming the cell sheets, 5) the ability to make contact on hard tissues and curved surfaces, and 6) the transplant is visible to the operators. dPDL cell sheets were transplanted into the surface of dental roots containing three-wall periodontal defects in an autologous manner, and bone defects were filled with porous beta-tricalcium phosphate (β-TCP). Cell sheet transplantation regenerated both new bone and cementum connecting with the well-oriented collagen fibers, while only limited bone regeneration was observed in the control group where cell sheet transplantation was not performed. These results suggest that PDL cells have multiple differentiation properties that allow for the regeneration of periodontal tissues composed of hard and soft tissues.

Next, we evaluated the safety and efficacy of PDL cell sheets in a one-wall infrabony defect model (Tsumanuma et al., 2011), which is considered to be a severe defect model (Kim et al., 2004). In this study, we also compared the differences in the periodontal healing of various cell sources. PDL cells, bone marrow derived mesenchymal stem cells, and alveolar periosteal cells were obtained from each animal, three-layered canine cell sheets were transplanted in an autologous manner, and bone defects were filled with porous β-TCP with 3% type I collagen gelto stabilize the graft shape. Eight weeks after transplantation, significantly more periodontal regeneration was observed in the newly formed cementum and well-oriented PDL fibers more in the PDL cell sheets group than in the other groups. These results indicate that PDL cell sheets combined with β-TCP/collagen scaffold serve as a promising tool for periodontal regeneration.

3.3. Optimization of human PDL cells

To protect human rights as subjects in clinical trials, the protocol of cytotherapy should be designed based on Good Clinical Practice (GCP) and Good Manufacturing Practice (GMP). Culturing hPDL cells from a single tooth is essential in performing our clinical trial. However, appropriate method for the extraction and expansion of hPDL cells are still not well understood. Thus, we determined the optimal method of isolation and expansion of hPDL cells and then examined their gene expression levels and differentiation potentials, and eventually validated the common characteristics of hPDL cells from 41 samples (Iwata et al., 2010). The hPDL cells were successfully extracted with collagenase/dispase, and then clonal proliferation was performed. Typically, 10 to 100 colonies were observed for a few days after the initial spreading. hPDL cells exhibit the ability to be highly proliferative when cultured at a low cell density. The cells were subcultured for 3 to 4 days, reaching one million cells in 2 weeks. Then, cells were spread on temperature responsive dishes to create a cell sheet in the presence of the osteoinductive medium. Cell sheets were harvested 2 weeks after spreading because the mRNA expression of osteogenic marker genes was strong after that period of time. Quality assurance tests were performed on at least 7 samples, and then the standard phenotypes of hPDL cell sheets were determined.

According to the GCP and GMP guidelines, hPDL cell sheets were created from three healthy volunteer donors at the GMP-grade Cell Processing Center (CPC) in our university (Washio et al., 2010). GMP-grade reagents and certified materials were used for culturing the hPDL cells. The safety and efficacy of "the product (hPDL cell sheets in this case)" was validated for a clinical trials. Prior to performing the cell culture, autologous serum was prepared from the donors. The hPDL cells were cultured under xeno-free conditions, and cell sheets were fabricated using the temperature responsive dishes. Culture sterility was confirmed using conventional tests. Safety was evaluated using the following tests: 1) the soft-agar colony-formation assay, 2) transplantation into nude mice, and 3) the karyotype test (Yoshida et al., 2012). The efficacy of the cell sheets was verified by transplantation with a dentin block into SCID mice. All of these tests revealed that hPDL cell sheets created at the CPC were safe and exhibited the ability to regenerate periodontal tissues. Another set of three hPDL cell sheets from healthy volunteer donors were created at the CPC to optimize the procedures.

3.4. The clinical trial

After approval on the 5th of January 2011, our clinical trial called "Periodontal regeneration with autologous periodontal ligament cell sheets" was initiated to treat patients presenting with the following ailments: 1) infrabony defects with a probing depth of more than 4 mm after the initial therapy, 2) radiographic evidence of infrabony defects, and 3) a redundant tooth that contains healthy periodontal tissue as a cell source. All patients provided written informed consent according to the GCP. Exclusion criteria included the following: 1) relevant medical conditions contraindicating surgical interventions (e.g., diabetes mellitus, cardiovascular, kidney, liver, or lung disease, or compromised immune system), 2) pregnancy or lactation, and 3) heavy tobacco smoking (more than 11 cigarettes a day). The primary out-

come of this trial is to evaluate the safety and efficacy of autologous transplantation of periodontal ligament cell sheets. As of the end of May in 2014, 10 cases of autologous PDL cell sheets were transplanted, and the healing process took place uneventfully.

4. Conclusion

The applications of cell sheet engineering for regenerative medicine are mentioned. Various types of cells have been examined and most of them improved the functions of recipients, suggesting that cell sheet engineering can be an alternative strategy for the therapy of tissue engineering. The implementation of robotic systems that allow the safe mass production of sterile cell sheets automatically, as well as further collaboration between researchers and medical professionals will make "cell sheet engineering" the leading edge solution for regenerative medicine (Elloumi-Hannachi et al., 2010).

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References

[1] Arauchi A, Shimizu T, Yamato M, Obara T, Okano T (2009). Tissue-engineered thyroid cell sheet rescued hypothyroidism in rat models after receiving total thyroidectomy comparing with nontransplantation models. *Tissue Eng Part A* 15(12):3943-3949.

[2] Asakawa N, Shimizu T, Tsuda Y, Sekiya S, Sasagawa T, Yamato M *et al.* (2010). Prevascularization of in vitro three-dimensional tissues created by cell sheet engineering. *Biomaterials* 31(14):3903-3909.

[3] Asano T, Takazawa R, Yamato M, Kageyama Y, Kihara K, Okano T (2005). Novel and simple method for isolating autologous mesothelial cells from the tunica vaginalis. *BJU Int* 96(9):1409-1413.

[4] Asano T, Takazawa R, Yamato M, Takagi R, Iimura Y, Masuda H *et al.* (2006). Transplantation of an autologous mesothelial cell sheet prepared from tunica vaginalis prevents post-operative adhesions in a canine model. *Tissue Eng* 12(9):2629-2637.

[5] Axelsson P, Lindhe J (1981). The significance of maintenance care in the treatment of periodontal disease. *Journal of Clinical Periodontology* 8(4):281-294.

[6] Burillon C, Huot L, Justin V, Nataf S, Chapuis F, Decullier E *et al.* (2012). Cultured autologous oral mucosal epithelial cell sheet (CAOMECS) transplantation for the treatment of corneal limbal epithelial stem cell deficiency. *Investigative ophthalmology & visual science* 53(3):1325-1331.

[7] Caton J, Nyman S, Zander H (1980). Histometric evaluation of periodontal surgery. II. Connective tissue attachment levels after four regenerative procedures. *J Clin Periodontol* 7(3):224-231.

[8] de Oliveira C, Watt R, Hamer M (2010). Toothbrushing, inflammation, and risk of cardiovascular disease: results from Scottish Health Survey. *Bmj* 340(c2451.

[9] Elloumi-Hannachi I, Yamato M, Okano T (2010). Cell sheet engineering: a unique nanotechnology for scaffold-free tissue reconstruction with clinical applications in regenerative medicine. *Journal of Internal Medicine* 267(1):54-70.

[10] Esposito M, Grusovin MG, Papanikolaou N, Coulthard P, Worthington HV (2009). Enamel matrix derivative (Emdogain(R)) for periodontal tissue regeneration in intrabony defects. *Cochrane database of systematic reviews* 4):CD003875.

[11] Flores MG, Hasegawa M, Yamato M, Takagi R, Okano T, Ishikawa I (2008a). Cementum-periodontal ligament complex regeneration using the cell sheet technique. *J Periodontal Res* 43(3):364-371.

[12] Flores MG, Yashiro R, Washio K, Yamato M, Okano T, Ishikawa I (2008b). Periodontal ligament cell sheet promotes periodontal regeneration in athymic rats. *J Clin Periodontol* 35(12):1066-1072.

[13] Haraguchi Y, Shimizu T, Yamato M, Kikuchi A, Okano T (2006). Electrical coupling of cardiomyocyte sheets occurs rapidly via functional gap junction formation. *Biomaterials* 27(27):4765-4774.

[14] Haraguchi Y, Shimizu T, Sasagawa T, Sekine H, Sakaguchi K, Kikuchi T *et al.* (2012). Fabrication of functional three-dimensional tissues by stacking cell sheets in vitro. *Nat Protoc* 7(5):850-858.

[15] Hasegawa M, Yamato M, Kikuchi A, Okano T, Ishikawa I (2005). Human periodontal ligament cell sheets can regenerate periodontal ligament tissue in an athymic rat model. *Tissue Eng* 11(3-4):469-478.

[16] Hata H, Matsumiya G, Miyagawa S, Kondoh H, Kawaguchi N, Matsuura N *et al.* (2006). Grafted skeletal myoblast sheets attenuate myocardial remodeling in pacing-induced canine heart failure model. *J Thorac Cardiovasc Surg* 132(4):918-924.

[17] Hoashi T, Matsumiya G, Miyagawa S, Ichikawa H, Ueno T, Ono M *et al.* (2009). Skeletal myoblast sheet transplantation improves the diastolic function of a pressure-overloaded right heart. *J Thorac Cardiovasc Surg* 138(2):460-467.

[18] Ishikawa I, Iwata T, Washio K, Okano T, Nagasawa T, Iwasaki K *et al.* (2009). Cell sheet engineering and other novel cell-based approaches to periodontal regeneration. *Periodontol 2000* 51(220-238.

[19] Iwata T, Yamato M, Tsuchioka H, Takagi R, Mukobata S, Washio K *et al.* (2009). Periodontal regeneration with multi-layered periodontal ligament-derived cell sheets in a canine model. *Biomaterials* 30(14):2716-2723.

[20] Iwata T, Yamato M, Zhang Z, Mukobata S, Washio K, Ando T *et al.* (2010). Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. *Journal of Clinical Periodontology* 37(12):1088-1099.

[21] Iwata T, Washio K, Yoshida T, Ishikawa I, Ando T, Yamato M *et al.* (2013). Cell sheet engineering and its application for periodontal regeneration. *J Tissue Eng Regen Med.*

[22] Kaneshiro N, Sato M, Ishihara M, Mitani G, Sakai H, Mochida J (2006). Bioengineered chondrocyte sheets may be potentially useful for the treatment of partial thickness defects of articular cartilage. *Biochem Biophys Res Commun* 349(2):723-731.

[23] Kanzaki M, Yamato M, Yang J, Sekine H, Kohno C, Takagi R *et al.* (2007). Dynamic sealing of lung air leaks by the transplantation of tissue engineered cell sheets. *Biomaterials* 28(29):4294-4302.

[24] Kanzaki M, Yamato M, Yang J, Sekine H, Takagi R, Isaka T *et al.* (2008). Functional closure of visceral pleural defects by autologous tissue engineered cell sheets. *Eur J Cardiothorac Surg* 34(4):864-869.

[25] Kim CS, Choi SH, Chai JK, Cho KS, Moon IS, Wikesjo UM *et al.* (2004). Periodontal repair in surgically created intrabony defects in dogs: influence of the number of bone walls on healing response. *Journal of Periodontology* 75(2):229-235.